THREE-STAGE ANALYSIS OF BLOOD COAGULATION*

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Ever since Morawitz published his classical review in 1905, it has been the custom to divide the blood-clotting process into two phases, or stages: (1) Prothrombin is converted to thrombin in the presence of calcium ions and certain factors derived from platelets and tissues. (2) Under the influence of thrombin, soluble fibrinogen is transformed to the insoluble fibrin.

This formulation was put forth as tentative, and it was generally appreciated that a two-stage theory could not give an adequate explanation of the mechanism. Several attempts were made to construct a three-stage framework on which further detail might be built. Morawitz (1904) suggested that prothrombin was first acted upon by thrombokinase, then by calcium. In somewhat similar fashion, Howell (1935) believed that cephalin freed prothrombin by removing heparin from it; then the prothrombin was activated directly by calcium. The 1940 schema of Wöhlisch postulated the conversion of prothrombin I to prothrombin II. Bordet (1919) had proposed that prothrombin must be "unmasked" in order to be readily convertible to thrombin; and his experiments had shown with particular clarity that some time-consuming reaction did, in fact, precede the development of thrombin. However, his data did not prove the assumption basic to all these three-stage theories, namely that the preliminary reaction was concerned with some preparatory change in the status of prothrombin.

Meanwhile another idea had been growing. Mellanby (1909) interpreted some of his data as indicating that serum contained an inactive form of thrombokinase. Closer to the point was the demonstration by Dale and Walpole (1916) that fowl plasma contained an inactive form of thrombokinase, from which active thrombokinase could be released by treating the plasma with chloroform or trypsin. The concept of a plasma precursor of thrombokinase was embodied in a three-stage theory of blood coagulation by Lenggenhager in 1936; and the proposed mechanism was explicitly diagrammed by him in 1940, in principle, as follows:

1. Prothrombokinase → thrombokinase in the presence of calcium.
2. Prothrombin → thrombin in the presence of calcium and thrombokinase.
3. Fibrinogen → fibrin in the presence of thrombin.

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Lenggenhager reported that prothrombokinase could be precipitated with a euglobulin fraction from platelet-free citrated plasma. He further stated that prothrombokinase was very labile and tended to become active even when gentle methods were used to precipitate the globulins. These observations were soon confirmed by Widenbauer and Reichel (1942).

The new three-stage theory offered Astrup (1944) a means of explaining the striking but enigmatic phenomenon of autocatalysis in blood coagulation. It had been known since the work of Arthus (1901) that thrombin was produced slowly, at first, then with increasing rapidity during the coagulation of whipped whole blood. Although by itself this observation might have been explained on the basis of two or more consecutive reactions, later work left little doubt that an autocatalytic or chain reaction was actually involved. Experiments described by Gratia (1922) and Fischer (1935) revealed that by serial seeding, an inciting agent for coagulation could be propagated through a series of otherwise stable samples of plasma. In quest of the underlying mechanism, Astrup searched for some evidence that thrombin catalyzed the activation of prothrombin, but his results were entirely negative (1939, 1944). He thereupon suggested that the autocatalytic effect was probably concerned with the activation of prothrombokinase, and indicated the chief impediments to further inquiry—the substances involved were labile and a reliable method of investigation had not been found.

The method of analysis described below was developed over a period of years, beginning with a study made in 1936 by Hellerman, Milstone, and Carnes. At that time it was found that the kinetics of prothrombin activation in crude preparations could not be described as a simple autocatalytic phenomenon, and that thrombin did not directly accelerate the activation of prothrombin. These unpublished findings suggested the same conclusions later reported by Astrup, and led to similar technical difficulties. The difficulties have now been overcome or circumvented sufficiently to allow the activation of prothrombokinase to be followed in a direct, quantitative manner.

The guiding idea in this work has been that if, indeed, the coagulation mechanism does embrace three distinct reactions, then it should be possible to carry out these primary reactions consecutively in separate test tubes. Moreover, as this goal was approached, new quantitative techniques should become feasible, and it should become easier to associate a given special effect, such as autocatalysis, with a particular primary reaction. Finally, it seemed of manifest importance to isolate and define the elusive "first" reaction, because the activation of prothrombokinase would naturally be a critical point in the physiologic control of the coagulation system.

Materials and Methods

Veronal-Buffered Saline—pH 7.4.—200 ml. 0.1 M sodium diethylbarbiturate
plus 144 ml. 0.1 M HCl
plus 0.9 per cent NaCl to 1,000 ml.

Stored in the refrigerator. The stock 0.1 M sodium diethylbarbiturate was also stored cold.

0.0275M Ca.—25 ml. 0.11 M $CaCl_2$ plus 75 ml. veronal-buffered saline.

0.0025M Ca.—10 ml. 0.0275 M Ca plus 100 ml. veronal-buffered saline.

$BaSO_4$ Suspension.—Prepared by the method of Tanturi and Banfi (1946) except that the $BaSO_4$ was collected and washed on a Büchner funnel.

Acetate Mixture for Prothrombin Preparation.—699 ml. 4 M sodium acetate
plus 69 ml. glacial acetic acid.
Glass electrode readings:
Undiluted, pH 5.45.
Diluted tenfold pH 5.00.

Buffer for Fibrinogen—pH 7.4.—67 ml. 0.1 M Na_2HPO_4.
13.4 ml. 0.1 M HCl.
100 ml. isotonic potassium oxalate (0.1272 M).
0.9 per cent NaCl to 1,000 ml.

Fibrinogen.—650 mg. bovine fraction I (Armour) was dissolved in 50 ml. buffer. 17 ml. saturated ammonium sulfate was added slowly. 15 minutes later the mixture was centrifuged lightly for 5 minutes, and the precipitate was immediately stirred thoroughly with 200 ml. 0.25 saturated ammonium sulfate. The residue was collected by centrifugation and dissolved in 75 ml. buffer. This fibrinogen preparation was stored at −17°C. Before use, it was thawed in a water bath at 30°C., whereupon the fibrinogen promptly and completely dissolved.

Frozen Euglobulin.—The source material for all plasma derivatives other than fibrinogen was a frozen euglobulin precipitate obtained from Armour and Co., of Chicago, through the cooperation of Dr. J. B. Lesh. Bovine blood was citrated, and the cells were removed by Sharples centrifugation. The plasma was diluted with ten volumes of cold tap water and the pH brought to 5.1 by addition of 1 per cent acetic acid. After settling overnight in the cold the supernatant fluid was discarded and the precipitate was shipped by air express, packed in dry ice. This material was still usable for the present type of work after a year's storage at −17°C. although even when first received, it contained some fibrin. 45.4 gm. represented 1 liter of citrated plasma.

Heated Globulin.—6 gm. Armour euglobulin was triturated with 40 ml. veronal-buffered saline and the pH brought back to 7.4 by cautious addition of 0.1 N NaOH. The undissolved material was removed by centrifugation, the pH readjusted to 7.4, if necessary, and the fluid transferred to four 12 ml. conical centrifuge tubes. The tubes were then kept in a 51°C. water bath for exactly 16 minutes and in cold tap water for 5 minutes. They were then allowed to stand at room temperature for ½ hour, during which the degree of flocculation increased. The heat-coagulated fibrinogen was then removed by centrifugation.

Prothrombokinase.—30 ml. heated globulin was mixed with 9 ml. $BaSO_4$ suspension in a 50 ml. centrifuge tube. The tube was stoppered with a clean rubber stopper and inverted at intervals for 20 minutes to keep the $BaSO_4$ suspended. The $BaSO_4$ was removed by centrifugation, and the adsorption procedure was repeated on the supernatant fluid, using 9 ml. of fresh $BaSO_4$ suspension. After 20 minutes, the $BaSO_4$

was centrifuged down, and the supernatant fluid was added to 270 ml. distilled water. One per cent acetic acid was added until a spot test with methyl red matched a spot standard of pH 5.0 acetate buffer plus methyl red. Heavy flocculation promptly followed, and the mixture was centrifuged. Glass electrode readings on the supernatant were usually between pH 5.2 and 5.5. The precipitate was stirred with 8 ml. veronal-buffered saline and the pH adjusted to 7.4 by cautious addition of 0.1 N NaOH. Prothrombokinase solutions were kept in the refrigerator when not in use, but were usually employed on the day of preparation. Several variations of this procedure were tried, both simpler and more elaborate, but most of the experiments were performed on this type of preparation. Prolonged centrifugation after the second adsorption with $BaSO_4$ made little difference in the behavior of the product.

Prothrombin.—According to recent reports prothrombin preparations of very high potency develop thrombin when incubated with calcium (Ferguson, Travis, and Gerheim, 1947) and may also show slight conversion in physiological saline (Ware, Guest, and Seegers, 1947). Therefore, mere high potency carries no guarantee that the prothrombin preparation is free of activating influences. For the present study, minimum contamination with activators was more important than high specific activity; and the work was facilitated by an abbreviated method for preparing a dilute prothrombin reagent which was stable in the presence of calcium ions.

The method was derived from that of Seegers, Loomis, and Vandenbelt (1945), modified and abbreviated as follows:—

136 gm. frozen euglobulin was dispersed in 250 ml. distilled water with a Waring blendor. To this was added 250 ml. cold oxalated salt solution (1.8 per cent NaCl: 1 per cent $K_2C_2O_4 \cdot H_2O$) and enough 0.1N NaOH to bring the pH to 7.4. Undissolved material was removed by centrifugation and the pH was readjusted to 7.4, if necessary. The solution was transferred to a one liter Erlenmeyer flask and kept in a 51°C. water bath for 30 minutes with frequent agitation of the flask and its contents. The flask was then put in cold tap water for a few minutes and then allowed to stand for $\frac{1}{2}$ hour at room temperature or several hours in the refrigerator. After this, the heat-coagulated fibrinogen was removed by centrifugation.

The heated globulin was stored in the refrigerator for 2 days or longer, during which it became less rapidly activatable by calcium. Any additional sediment was removed by centrifugation; and 100 ml. of globulin solution was treated with 28 ml. $Mg(OH)_2$ suspension prepared by the method of Seegers *et al.* (1945). After 15 minutes at room temperature the $Mg(OH)_2$ was collected by centrifugation in lusteroid tubes, and then mixed with 100 ml. distilled water at room temperature. The mixture was thoroughly stirred for 15 minutes, with particular care to break up all clumps, and then centrifuged. After three such washes, the $Mg(OH)_2$ sediment was stirred thoroughly with 100 ml. acetate mixture. Any material not dissolved in 1 hour was centrifuged out. The solution was dialyzed in a rocking dialyzer for $1\frac{1}{2}$ hours, during which 18 liters of 0.9 per cent NaCl were run through the dialyzer. The solution was then dialyzed in the refrigerator against several changes of veronal-buffered saline, over a period of 24 hours. At this time, the prothrombin solution was at pH 7.4, and after centrifuging to remove a slight amount of precipitate, the solution was stored in the freezer.

Lipoid Thromboplastin.—That fraction of bovine brain which was soluble in ether, but not in acetone.

To prepare a stock thromboplastin suspension, 300 mg. of the solid was triturated with 3 ml. veronal-buffered saline. This suspension, when diluted to 10^{-4} in a prothrombin activation mixture, still accelerated the appearance of thrombin. It lost very little of its activity when heated at 60°C. for 10 minutes.

Determination of Coagulation Time.—In an earlier paper (Milstone, 1942), a method was described for assaying thrombin preparations of high potency, using a standard thrombin preserved in glycerin. In the present work, the demands of the situation were different. Here, it was of chief importance to determine how much of a given prothrombin solution had been converted to thrombin at any given moment; in other words, to estimate thrombin in the presence of prothrombin at a sharply defined time, and further to make such estimates at several consecutive intervals. Therefore, in this study, certain operations were avoided, which although promising greater volumetric accuracy would have made timing less precise or consecutive repetition more difficult.

0.1 ml. of the solution to be assayed was carefully measured in the last 0.1 ml. of a 1.0 ml. serological pipette. With a finger over the top of it, the pipette was introduced into a 10 × 75 mm. pyrex tube containing 0.3 ml. oxalated fibrinogen and poised with its tip a few millimeters above the level of the fibrinogen. At the moment when a running stop-watch passed the zero mark, the thrombin was blown into the fibrinogen with sufficient force to cause rapid mixing. (The effectiveness of this procedure was checked by blowing 0.1 ml. of a dye solution into 0.3 ml. fibrinogen.) The blowing was rapidly repeated 2 or 3 times and mixing was promptly continued for another 5 seconds by rapid oscillation of the tube. The coagulation process was followed by repeatedly tilting the tube; and the coagulation time was taken as the interval between the addition of thrombin (zero on the stop-watch) and the instant when the clot would hold its position with the tube inverted. Coagulation times longer than 25 minutes tended to be erratic.

Estimation of Thrombin in the Presence of Prothrombin

With the present materials and methods, it was found that the speed of coagulation was directly proportional to the concentration of added thrombin, within the limits of experimental error. To verify this relationship, a thrombin solution was diluted with the routine buffer-calcium medium, and the coagulation times were determined when each of the dilutions was tested with oxalated fibrinogen. The results are represented by the open circles in Fig. 1, where the reciprocal of the coagulation time, *i.e.* the speed of coagulation, is plotted against the known relative concentration of added thrombin. These data, together with others, showed that deviations from the straight-line function were haphazard and not greater than would have been expected from the experimental error, an appreciable share of which was due to variations in pipettes. Hence, it was at least as accurate to use the simple straight-line relationship as it would have been to use any one empirical curve obtained at a particular time.

Although any number could have been used to convert coagulation time to relative speed of coagulation, 3,000 was chosen because it was convenient to have a coagulation time of 30 seconds represent a thrombin concentration of "100," or, in algebraic form, Relative thrombin concentration $= \frac{3{,}000}{\text{C.T.}}$. On this basis a solution which clotted fibrinogen in 60 seconds would be said to have a relative thrombin concentration of "50," etc. Reducing the present results to terms of some arbitrary standard thrombin would not have materially increased the significance of the principles demonstrated, and therefore the assay

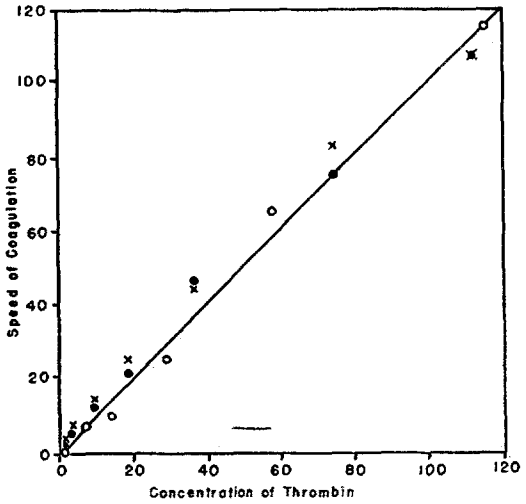

FIG. 1. Relation between speed of coagulation and concentration of thrombin. Open circles, dilutions of thrombin in buffer—0.0025 M Ca. X's, rapid dilutions of thrombin in prothrombin—0.0025 M Ca. Solid circles, rapid dilutions of thrombin in buffer—0.0025 M Ca. 0.1 ml. thrombin dilution plus 0.3 ml. oxalated fibrinogen. Speed of coagulation = 3,000/coagulation time.

values are not meant to bear any definite relationship to various thrombin units which have been employed. However, they will be used in any given experiment, as if they had the connotation "100 units per ml. thrombin solution," etc.

It was further shown that the thrombin assay values were not appreciably affected by the presence of prothrombin. For example, a mixture of 20 per cent thrombin and 80 per cent prothrombin gave in the usual test a coagulation time about the same as that given by a mixture of 20 per cent thrombin and 80 per cent buffer-calcium medium. From this it might be expected that at the moment when a full strength prothrombin had become 20 per cent activated, it, too, would give the same coagulation time, reflecting the fact that 20 per cent of it was thrombin at the time of sampling. In the main this is correct,

with a minor complication which is partly taken into account in the following test, and need not be discussed until later.

The experiment was performed on crude preparations, so that a certain amount of activator material would be brought into the mixture by both the prothrombin and the thrombin. Thrombin was diluted with prothrombin in such a manner that the total concentration of thrombin plus prothrombin was always the same and the concentration of calcium was maintained at 0.0025 M in all cases. These manipulations entailed the admixture of prothrombin, calcium, and activator material, and were therefore performed rapidly; and each mixture was assayed for thrombin immediately. The results are portrayed by the ×'s in Fig. 1, and are to be compared with similarly rapid dilu-

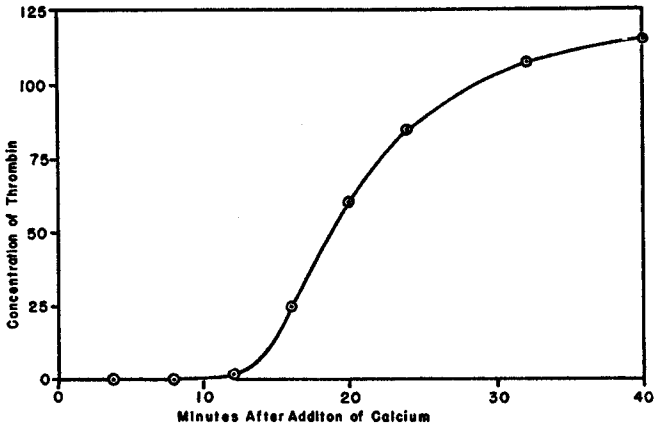

FIG. 2. Activation of crude prothrombin. 0.1 ml. heated globulin and 0.9 ml. veronal-buffered saline, plus 0.1 ml. Ca 0.0275 M.

tions of thrombin in buffer-calcium medium, represented by solid circles. It can be seen that, despite the disadvantages taken, the presence of prothrombin, plus the amount of activator material included, did not appreciably alter the values of the thrombin assays.

The Latent Period in the Activation of Crude Prothrombin

After fibrinogen has been removed from a crude euglobulin solution by heat coagulation at 51°C., the remaining solution still contains everything necessary to produce thrombin upon the addition of calcium. Fig. 2 illustrates the commonly observed pattern of activation. Calcium was added to the diluted globulin, and at the intervals indicated, samples were assayed for thrombin. In this experiment, as in all others where samples were transferred at specified times, the time of sampling was not that at which the sample was removed from the first tube, but the moment when it was added to the next reagent. As shown by Fig. 2, no thrombin was found in the samples taken at 4 and 8

minutes, but a small amount was detectable at 12 minutes. Then, suddenly, thrombin developed with increasing velocity and levelled off toward a plateau value. Activation curves for different crude globulin preparations were found to vary a great deal, especially in the duration of the latent period. However, the general pattern was consistent, and proved to be independent of the presence or absence of fibrinogen or of the manner in which fibrinogen was removed. Similar curves were obtained when the prothrombin was activated in the presence of sufficient fibrinogen to form a solid clot, and also when the crude globulin had been previously defibrinated by the addition of a minimal quantity of oxalated thrombin. However, it was obvious that the activation pattern depended on some factor in addition to prothrombin and calcium. In a previous study (Milstone, 1942) it was noted that it became more difficult to activate prothrombin by the mere addition of calcium as the prothrombin was purified. At that time, it was found (unpublished data) that the condition for normal activation could be restored by adding a small amount of a by-product fraction; and it was tentatively assumed that the additional factor was the precursor of thrombokinase. As recounted above, such a factor had previously been recognized by others. In conformity with what appears to be the oldest and most widely prevalent usage, this factor will now be called prothrombokinase, bearing in mind that the preparations herein studied may contain an activator complex with more than one significant component.

If now two solutions were available, one containing prothrombin, and the other containing prothrombokinase, a combination of these solutions might duplicate the activation pattern depicted by Fig. 2. If so, it should be possible to inquire what was going on during the latent period. If each factor were separately incubated with calcium, it could be determined whether the preliminary interval was occupied by a reaction involving calcium and one or the other of the two thrombin-producing factors.

Adsorption techniques proved useful in separating the two factors, since prothrombokinase appeared to be less avidly adsorbed by such substances as magnesium hydroxide or barium sulfate than was prothrombin. It was possible to remove all but a trace of prothrombokinase from prothrombin by thoroughly washing the magnesium hydroxide on which the prothrombin had been adsorbed. Deterioration of prothrombokinase during the procedure may well have contributed to the success of the preparation. Starting with a crude euglobulin solution, prothrombokinase was freed of most of the accompanying prothrombin by two adsorptions with barium sulfate. When calcium was added to an appropriate mixture of the prothrombokinase and prothrombin reagents, the activation curve was much like that for crude globulin. This suggested that the effect of the procedures had been essentially that of separation, and that otherwise nothing significant had been introduced or lost.

When the prothrombin reagent was incubated with calcium first, and then

prothrombokinase added, the latent period was sometimes a little shorter than that of the control, but it was always clearly in evidence. In contrast, it was possible to abolish the latent period completely by pre-incubation of prothrombokinase with calcium. From this, it appeared that the latent period was concerned with the activation of prothrombokinase under the influence of calcium.

Further examination of the slight effect obtained by pre-incubation of prothrombin with calcium showed that, as a batch of such material stood at room temperature, samples taken from it at intervals first gave slightly shorter latent periods upon activation with prothrombokinase. As incubation of the prothrombin-calcium mixture continued, however, samples taken from it gave slightly longer latent periods. These findings were taken to indicate that a slight amount of prothrombokinase still remained in the prothrombin reagent. This contaminant became active in the presence of calcium and contributed to a slight shortening of the latent period. But, as incubation was continued, the active trace of thrombokinase deteriorated and the latent periods then became longer. All the results which follow are in harmony with this interpretation.

Kinetics of Prothrombin Activation

Not only did prolonged incubation of the prothrombin reagent with calcium fail to abolish the latent period; it also failed to result in a significant production of thrombin. Consequently, it became convenient to use a mixture of prothrombin and calcium as a single reagent. Often such prothrombin-calcium reagents were stored for several days in the refrigerator, during which they developed no significant amount of thrombin (no coagulation in 1 hour) and continued to function satisfactorily as prothrombin.

A steady prothrombin-calcium reagent could be used to define the changing status of a prothrombokinase-calcium mixture, because the latter would be responsible for any change in the kinetics of thrombin production.

Calcium was added to prothrombokinase, and at intervals, 0.1 ml. samples were transferred to 0.9 ml. samples of prothrombin-calcium reagent, the concentration of calcium being 0.0025 M throughout. Then, in each case, a prothrombin activation curve was obtained as already described for Fig. 2. Preliminary experiments at room temperature showed that the activation of prothrombokinase was too rapid to permit such detailed study and that subsequent deterioration was likewise rapid. Therefore, the activation of prothrombokinase was performed in a water bath kept in the refrigerator. Further orienting experiments revealed that when the activation of prothrombin was also performed in the cold, these latter reactions were very slow and the procedure was cumbersome. Therefore, prothrombokinase was activated in the cold, whereas both the activation of prothrombin and the assay of thrombin

by coagulation of fibrinogen were carried out at room temperature. It will be noted at this point that we are already dealing with a three-stage procedure, in which the experimental steps correspond to the three theoretical stages: (1) activation of prothrombokinase; (2) activation of prothrombin; (3) coagulation of fibrinogen (Milstone, 1947).

Some of the results are presented in Fig. 3, which focuses attention on the early portions of the prothrombin activation curves. In this experiment, the

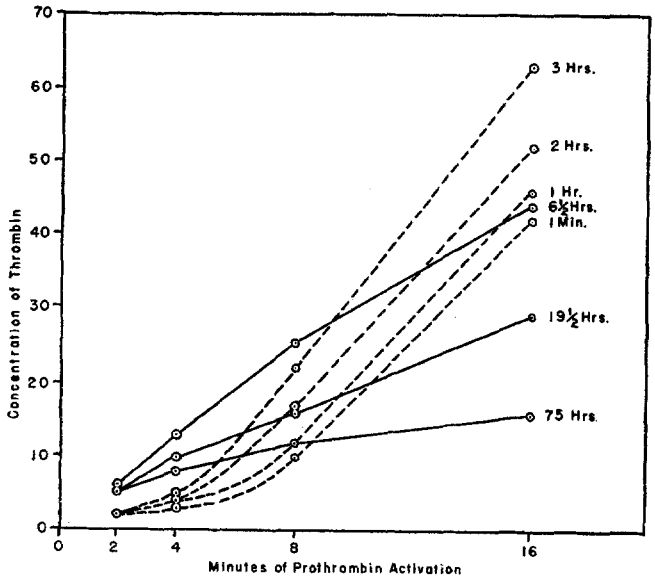

FIG. 3. Successive prothrombin activation curves obtained with samples of prothrombokinase in progressive stages of activation (broken lines), and decline (solid lines). 5.0 ml. cold prothrombokinase plus 0.5 ml. Ca 0.0275 M. Kept cold. 20 ml. prothrombin plus 2 ml. Ca 0.0275 M. At room temperature. The time at the right end of each curve is the age of the prothrombokinase-calcium mixture when 0.1 ml. of it was transferred to 0.9 ml. prothrombin-calcium mixture.

latent period was unusually short at the outset, so that its subsequent obliteration was not as spectacular as in some cases. Nevertheless, the data collected during the run offer a detailed description of the manner in which the latent period is progressively shortened and finally abolished.

During the first 3 hours of prothrombokinase activation, the curves for activation of prothrombin showed that the former was steadily gaining in capacity to activate prothrombin rapidly. In addition, all the early curves, portrayed by broken lines, had a sharp upsweep, indicating that the rate of thrombin production was being accelerated during the course of the reaction. In these curves, furthermore, the *initial rate* of prothrombin activation was close to

zero at first, and then slowly increased as prothrombokinase was activated for 3 hours. In the prothrombin activation curve obtained at $6\frac{1}{2}$ hours, the circumstances were quite different. There, activation of prothrombin began with maximal velocity; and the rate of activation was never accelerated thereafter, but rather it steadily diminished. Despite the fact that the $6\frac{1}{2}$ hour curve started out so much faster than the earlier curves, it soon fell below them. This suggested that, while the prothrombokinase had been converted to the active form, some activity had been lost through deterioration or side reactions, so that the sum of active plus potential thrombokinase was less than when the prothrombokinase-calcium mixture was fresher. That the thrombokinase was actually deteriorating was demonstrated by the curves obtained at $19\frac{1}{2}$ hours and 75 hours.

In view of the possibly enzymatic nature of thrombokinase, it was of particular interest to inquire how closely the prothrombin activation curves approached the theoretical curves for a unimolecular reaction. In such a case, the function $\log \frac{A}{A-a}$ should give a straight line when plotted against activation time t, where A equals the maximum thrombin value at the plateau of the activation curve and a represents the amount of thrombin at time t. In such fashion, data of Fig. 3 plus some additional data collected during the experiment are replotted in Fig. 4. There it is seen that curves obtained with partially activated prothrombokinase turn upwards, whereas those curves depicting the activation of prothrombin by ripe thrombokinase closely approximate the straight lines characteristic of unimolecular reactions.

This experiment also gives further evidence that thrombin does not directly catalyze the activation of prothrombin. If it did, all the curves in Fig. 3 would be expected to turn upwards, and none of the curves in Fig. 4 should so closely approximate the unimolecular form.

Estimation of Thrombokinase

As just demonstrated, the activation of prothrombokinase can be followed by comparing the prothrombin activation curves resulting from serial tests. It could have been followed more simply, in the experiment of Fig. 3, by determining how much prothrombin was activated in the first 4 minutes in each test. As is evident from Fig. 3, the activation at the 4 minute intercepts of the curves became progressively greater as the prothrombokinase was incubated with calcium for $6\frac{1}{2}$ hours. In the later work, more concentrated solutions of prothrombokinase were used, with the result that 2 minute intercepts were more appropriate; otherwise the technique was the same. While this device furnished a means of following the activation of prothrombokinase, it was further desirable to reduce the data to a quantitative basis.

To accomplish this, a solution of prothrombokinase was mixed with calcium

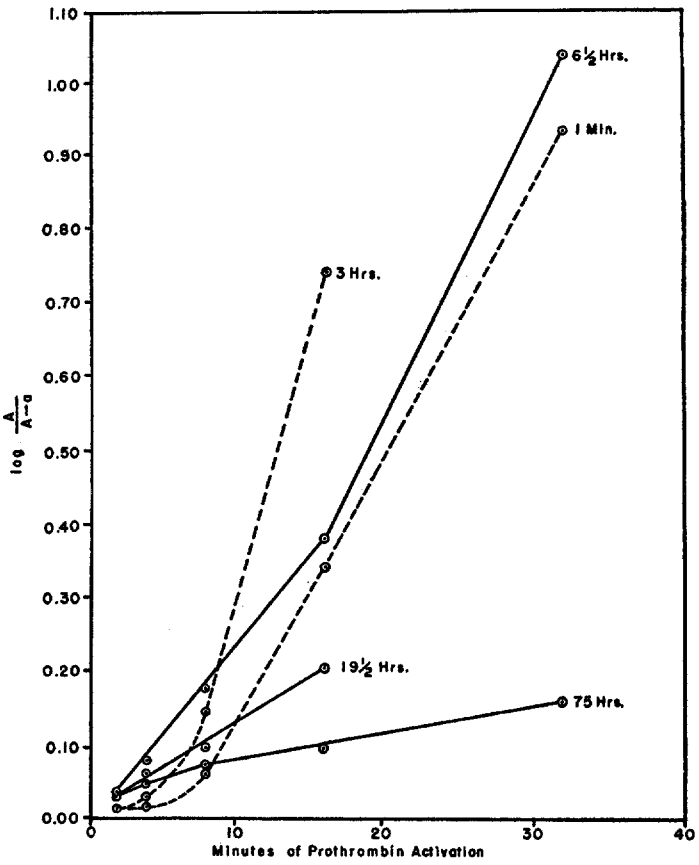

FIG. 4. Activation of prothrombin plotted as a unimolecular reaction. Same experiment as Fig. 3.

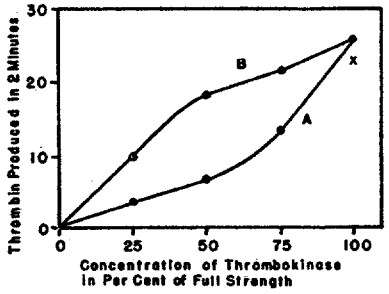

FIG. 5. Activation of prothrombin with increasing concentrations of thrombokinase. *A*, dilutions of thrombokinase in buffer—0.0025 M Ca. *B*, dilutions of thrombokinase in prothrombokinase—0.0025 M Ca.

in the cold, and the course of activation followed by the above method. When the activity had fully developed, the thrombokinase was promptly diluted with buffer-calcium solution. For each dilution, it was determined experimentally how much prothrombin would be activated by it in 2 minutes. The results are plotted in Fig. 5, and demonstrate that the concentration of added thrombokinase determined the initial rate of prothrombin activation. This would be expected if thrombokinase were an enzyme. However, the empirical relationship is sufficient basis for the quantitative estimation of thrombokinase.

As previously mentioned, the initial rate of prothrombin activation is practically nil when prothrombokinase is used, in place of thrombokinase. This would offer a simple way of estimating the active form in the presence of the precursor, for any initial activation would be due entirely to thrombokinase. The main difficulty is that the activation of prothrombokinase continues, even after its tenfold dilution in prothrombin, no doubt aided by the change from refrigerator to room temperature. Merely running the prothrombin activation in the cold would not solve this problem, because a much longer time would be required for the production of measurable thrombin, and this in turn would allow more time for additional activation of prothrombokinase. Perhaps this will be less troublesome at a later date, when it becomes convenient to use a more concentrated prothrombin "substrate."

It was considered that the activation of prothrombin might be much less sensitive to a decrease in calcium concentration than was the activation of prothrombokinase. If the prothrombokinase-calcium mixture were added to nine volumes of a prothrombin solution that contained no added calcium, then the calcium would be diluted along with the prothrombokinase; and perhaps the activation of prothrombokinase would stop, while the production of thrombin proceeded. But, it was found that the concentration of calcium was important for both reactions. Activation of prothrombin was very slow with 0.00025 M calcium. This conclusion was verified by restoring the calcium to the usual concentration just before the thrombin assays, thus eliminating the possibility that the apparent retardation of thrombin formation was due merely to an effect on the measurement of thrombin.

For the present, it seemed best to make an allowance for the production of extra thrombokinase, in lieu of preventing it. Cold thrombokinase was diluted with cold prothrombokinase in such a way that the total concentration of the two was constant, and calcium was 0.0025 M throughout. The initial rate of prothrombin activation was measured for each dilution, and the results plotted as curve B in Fig. 5. The higher course of curve B, as compared with curve A is largely attributable to the appearance of additional thrombokinase during the 2 minutes of prothrombin activation.

The assumption was then made that an artificial mixture of half thrombokinase and half prothrombokinase would be equivalent to a solution of prothrom-

bokinase that had become half-activated, and similarly for other proportions of active to precursor form. This seemed plausible, provided the standardization was made anew for each set of materials at the time they were being studied. With the exception of this assumption, the standardization was empirical, and relative to the peak activity shown by the particular thrombokinase.

As a result of their empirical nature, the standard curves controlled another possible source of error. Quick (1940) and Owren (1947) have described conditions under which production of thrombin seems to continue for a brief time after oxalation. In the experiment of Fig. 1, such did not occur to a significant extent. There the amount of activator material was about twice that present in the experiments of Figs. 2, 3, and 4, but only about half that present in later experiments. If, with higher concentrations of thrombokinase, this complication did reach a significant level, it was controlled by the empirical curves. In using them, the over-all effect of the unknown was compared to that of the standard under the same conditions.

Now that it was feasible to estimate thrombokinase in the presence of its precursor, the activation of prothrombokinase could be followed in a quantitative manner.

Kinetics of Prothrombokinase Activation

Table I presents the details of a three-stage procedure, as used in studying the activation of prothrombokinase. The first stage was represented by a prothrombokinase-calcium mixture which was kept in a water bath at 6°C. Both the prothrombokinase and the calcium solution used to activate it were cold at the start, likewise all glassware that came in contact with these solutions had been prechilled by storage in closed containers in the refrigerator. The second stage was represented by a series of tubes containing the prothrombin-calcium reagent, and the third stage by a series of tubes containing oxalated fibrinogen. Although these latter materials were also refrigerated during the $7\frac{1}{2}$ hour experiment, they were warmed to room temperature before use in the test.

The gross effects to be observed in such a procedure were so striking that it was an easy matter to follow the reaction while it was in progress, by mere inspection of the raw data. These are listed in the first and third columns of Table I. There it can be seen that 2 minutes after calcium was added to prothrombokinase, a sample of this mixture was incubated with prothrombin for 2 minutes, with the result that not enough thrombin was formed to clot fibrinogen in 3600 seconds. At 120 minutes, the prothrombokinase-calcium mixture had ostensibly changed so little that a similar series of operations resulted finally in a 3,000-second coagulation time. But at 150 minutes, the change in the prothrombokinase suddenly became evident. It was now sufficiently active

so that in 2 minutes with prothrombin, it brought forth enough thrombin to produce a clot in 465 seconds. Similar tests at 180 and 210 minutes gave evidence that the prothrombokinase was approaching full activation, as anticipated from previous experience with this type of system. Therefore, when some further increase in activity was recorded at 240 minutes, it was assumed that thrombokinase activity was near its peak. This was the time to set up standard mixtures for the estimation of relative concentration of thrombokinase.

TABLE I

Data Obtained with the Three-Stage Procedure

Tube 1 Activation of prothrombokinase	Second series of tubes Activation of prothrombin	Third series of tubes Coagulation of fibrinogen	Tube 1
0.1 ml. of 0.0275 M Ca added to 1.0 ml. prothrombokinase	At time t 0.1 ml. transferred from tube 1 to 0.9 ml. of prothrombin containing 0.0025 M Ca	After 2 min. activation 0.1 ml. transferred from second series tube to 0.3 ml. oxalated fibrinogen	Relative concentration of thrombokinase at time t
Min. after addition of Ca, t	Thrombin produced in 2 min. 3,000/C.T.	Coagulation time	Per cent of full strength
		sec.	
2	<0.9	>3600	0
30	1.3	2400	3
60	<0.9	>3600	0
90	<0.9	>3600	0
120	1.0	3000	3
150	6.5	465	17
180	20.0	150	62
210	20.0	150	62
240	26.1	115	100
330	23.1	130	95
450	0.1 ml. transferred from tube 1 to 0.9 ml. buffer-calcium	1320	

The activation of prothrombokinase was carried out at 6°C.; the other two reactions were carried out at room temperature, 29°C.

The mixtures were prepared according to the considerations previously discussed, and the technical details are given in Table II. It will be noticed that the entire process of preparing a standard mixture in the cold required 80 seconds. The first 40 seconds, during which prothrombokinase was in contact with calcium were insignificant, because activation starts off so slowly at 6°C. Even the ensuing 40-second contact of thrombokinase with prothrombokinase could have made little difference, again because such mixtures change so slowly in the cold. The results of Table II were actually those which have been presented in Fig. 5.

After these standard mixtures were tested, another test was performed on the original prothrombokinase-calcium mixture which was now 330 minutes old. As shown in Table I, and also by the × in Fig. 5, a slight deterioration had occurred. In a further control, a sample of the thrombokinase was added to buffer-calcium solution instead of prothrombin, and 2 minutes later the mixture was assayed for thrombin, giving a clot in 1320 seconds. This indicated that

TABLE II

Standard Mixtures

Relating Observed Rate of Prothrombin Activation to:
A. Relative Concentration of Added Thrombokinase
B. Per Cent of Thrombokinase in a Thrombokinase-Prothrombokinase Mixture

Tube 1		Tube 1		Tube 3	Tube 2
Relative concentration of thrombokinase		Thrombokinase at peak activity*	Buffer-Ca mixture	Coagulation time	Thrombin produced in 2 min.
		ml.	*ml.*	*sec.*	
A	25	0.25	0.75	840	3.6
	50	0.50	0.50	440	6.8
	75	0.75	0.25	220	13.6
	100	Undiluted		115	26.1
			Fresh‡ prothrombokinase-Ca mixture *ml.*		
B	25	0.25	0.75	300	10
	50	0.50	0.50	165	18.2
	75	0.75	0.25	140	21.4
	100	Undiluted		115	26.1

* Peak activity—240 to 330 minutes after Ca added—*cf.* Table I.

‡ These mixtures were each 40 seconds old at the time they were added to the thrombokinase. A fresh prothrombokinase-Ca mixture was prepared for each thrombokinase-prothrombokinase mixture; and all were prepared cold. The thrombokinase-prothrombokinase mixtures were 40 seconds old at the time a 0.1 ml. sample was added to 0.9 ml. prothrombin containing 0.0025 M Ca. Otherwise the general procedure was the same as that outlined in Table I, and the same materials were used.

even 2 adsorptions with barium sulfate had not removed all the prothrombin from the prothrombokinase reagent, and that the residual prothrombin became active along with the prothrombokinase. On a quantitative basis, the resulting error in the estimation of thrombokinase was not great, not only because of the small difference involved, but also because use of the standard thrombokinase-prothrombokinase mixtures would be expected to allow for this factor.

Another control, performed 6 hours after the beginning of the experiment showed that the prothrombin-calcium reagent still behaved quite the same as it

had at the beginning. When a fresh prothrombokinase-calcium mixture was tested with it, not only did a zero value for initial rate of activation result, but, further, the activation curve through the first 16 minutes was practically the same as that which had been obtained with the original prothrombokinase-calcium mixture at the start. Although this behavior was usually approached by the prothrombin-calcium reagents used in these later experiments, it may be stated that other types of prothrombin preparations have been found to give highly variable activation curves as they aged, even though they did not produce detectable thrombin when incubated with calcium. Both evidences of stability are required in order that the prothrombin be dependable in a three-stage procedure.

The coagulation times listed in the third column of Table I were converted to values for thrombin, as listed in the second column. From the amount of thrombin produced in 2 minutes, values for relative concentration of thrombokinase were derived by use of Fig. 5. These, listed in the last column of Table I, give a quantitative expression for what has already been observed in a general way—that the activation of prothrombokinase proceeded imperceptibly for the first 2 hours, rapidly increased during the 3rd hour, and leveled off at the end of the 4th. The data are plotted in Fig. 6 where the circles represent experimental points and the curve is the theoretical for an autocatalytic reaction. From one of the calculated parameters presented with Fig. 6, it may be concluded that a fair approximation would result if the entire process were treated as a simple autocatalytic reaction in which 0.006 per cent of the total potential activity was in the active form at the start. That the reaction actually is complicated by deterioration and possibly by side reactions has been mentioned above.

Further evidence that the activation of prothrombokinase involves an autocatalytic or chain reaction soon appeared. A fresh mixture of prothrombokinase and calcium was seeded with an additional 5 per cent of fresh thrombokinase. While the seeding increased the initial percentage of active form, it did not affect the total concentration of thrombokinase plus prothrombokinase, or the concentration of calcium. Followed in the cold, the activation of the seeded prothrombokinase reached its peak at the time the curve for the unseeded control was just beginning to leave the base line.

Activation of Prothrombokinase at Room Temperature

The activation of prothrombokinase can also be studied at room temperature. For exploratory work, this procedure is rapid and convenient. Here, the use of thrombokinase-prothrombokinase standards is not feasible, because at room temperature such mixtures change too fast in relation to the time required to make the necessary manipulations. Nevertheless, an arbitrary conversion chart can be made by employing a straight line instead of curves like those of

Fig. 5. The effect of this substitution is to distort the shape of the curves, and to shift them only slightly along the time axis. This was verified by replotting the data of Fig. 6, using a straight line to convert observed rate of prothrombin activation to concentration of thrombokinase.

Whereas such an approximation was useful in enabling a graphic representation of results, the general effects to be described were large and clearly dis-

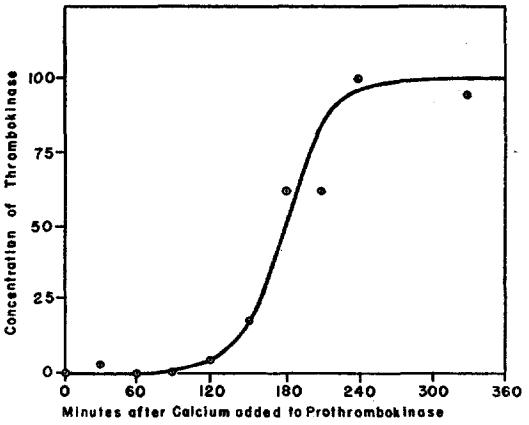

FIG. 6

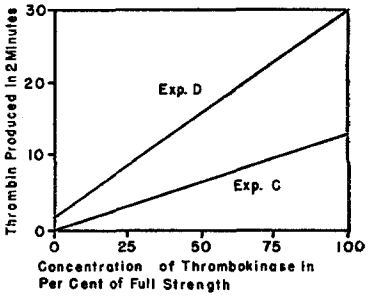

FIG. 7

FIG. 6. Activation of prothrombokinase at 6°C. Circles, experimental points. Smooth curve, calculated from equation for simple autocatalytic reaction:

$$KA_c t = 2.3 \log \frac{A(A_c - A_o)}{A_o(A_c - A)} \qquad \text{(Kunitz and Northrop, 1936)}$$

where

K = 0.000543
A_c = 100 per cent of peak activity
A_o = 0.006 per cent of peak activity
A = per cent of peak activity at time t

FIG. 7. Arbitrary chart for converting initial rate of thrombin formation to relative concentration of thrombokinase.

cernible from the raw data. Table III, which partitions the observed results from the calculated data, illustrates this point. In Experiments C and D, large differences in results obtained, bear witness to the fact that seeding accelerated the activation of prothrombokinase.

Experiment D is also of interest because it presents an example of another complication occasionally encountered. Here the initial rate of thrombin production was measurably above zero, no matter how fresh the mixture of prothrombokinase and calcium. This may have been due to the contamination of the prothrombin-calcium reagent with a trace of thrombokinase, larger than usual. The complication was treated as a blank when the arbitrary lines were

drawn for converting initial rate of prothrombin activation to concentration of thrombokinase. The largest blanks encountered in any three-stage experiments were those of Experiment D of Table III, and the early experiment presented in Fig. 3.

After the coagulation times of Table III had been converted to values for initial rate of thrombin production, an arbitrary graph was drawn for each experiment, as illustrated in Fig. 7. For Experiment C the line was drawn from

TABLE III

Activation of Prothrombokinase at Room Temperature, Followed by Three-Stage Procedure. Acceleration by Seeding with One-Eleventh Volume of Thrombokinase

Observed data					Calculated data							
Tube 1	Third series tubes				Second series tubes				Tube 1			
Minutes after Ca added to prothrombokinase, t	Coagulation time, sec.				Amount of thrombin produced in 2 min., 3,000/C.T.				Relative concentration of thrombokinase at time t			
	Exp. C Control	Exp. C Seeded	Exp. D Control	Exp. D Seeded	Exp. C Control	Exp. C Seeded	Exp. D Control	Exp. D Seeded	Exp. C Control	Exp. C Seeded	Exp. D Control	Exp. D Seeded
1	>3600	1440	1860	570	<0.9	2.1	1.6	5.3	0	18	0	13
2	>3600	570	1800	180	<0.9	5.3	1.7	17	0	42	0	53
5	>3600	270	1620	115	<0.9	11	1.9	26	0	85	1	87
8	1920	270	270	105	1.6	11	11	29	13	85	33	97
12			100				30				100	
16			110				27				90	
20	240		140		13		21		100		68	

Tube 1, Second series tubes, and Third series tubes have the same significance as in Table I, except for the temperature difference.

In seeding the mixtures, the thrombokinase was added to the prothrombokinase 15 to 30 seconds after the calcium was added.

Any sample of prothrombokinase was always seeded with a sample from its own batch, freshly activated.

the blank value of zero to the peak value of 13. For Experiment D, the line was drawn from 1.6 to 30. From this chart were derived the values for relative concentrations of thrombokinase which are entered in Table III. They show, in numerical fashion, that seeding with thrombokinase hastened the activation of prothrombokinase.

The three-stage procedure at room temperature was then used for several exploratory experiments. Fig. 8 presents an example. First a batch of prothrombokinase was activated by the addition of calcium and the conversion process was followed as just described. The development of thrombokinase is plotted in Fig. 8 as "first control." When peak activity was reached, a portion was heated at 60°C. for 10 minutes, then promptly cooled to room temperature.

Eighty-five minutes after the start of the first activation, two parallel tests were run, starting 1 minute apart. One of these was seeded with one-eleventh volume of heated thrombokinase, the other with unheated. Four hours after the start of the first control, a final control was run on unseeded prothrombokinase.

It is evident from Fig. 8 that both thrombokinase and its precursor were labile under the conditions of the experiment. In each test, thrombokinase declined rapidly from its peak value. The activation curve for the final control was different from that for the first control. This indicated that, in 4 hours, a detectable change had occurred in the prothrombokinase, even without the addition of calcium. Indeed, prothrombokinase solutions were regularly so

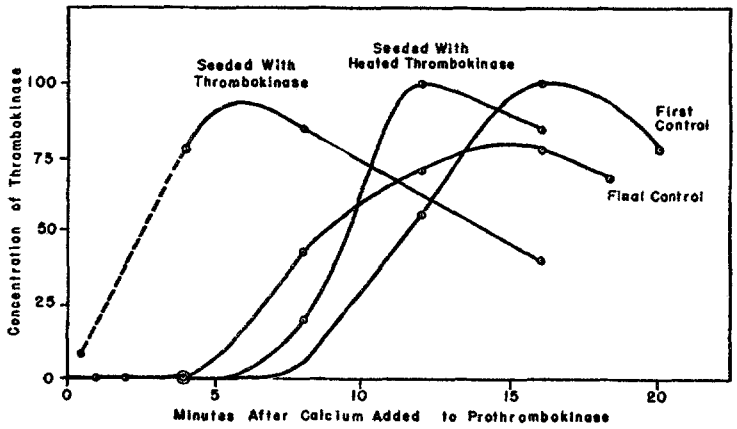

FIG. 8. Three-stage tests comparing heated with unheated thrombokinase in their capacity to accelerate the activation of prothrombokinase. In seeding the mixtures one-eleventh volume of thrombokinase was added 30 seconds after calcium.

unstable that they were used only on the day of preparation, with rare exceptions. It was also necessary, in order to achieve comparable conditions, to run each test at the same time as that to which it was to be compared.

In Fig. 8, it is seen that heated thrombokinase caused a slight, doubtful acceleration, as compared with the definite acceleration effected by the unheated thrombokinase. Additional tests showed that the presence of heated thrombokinase did not prevent an inoculum of the unheated agent from hastening the activation process. Finally, the heated sample was found to cause very slow production of thrombin as compared with an unheated sample of the same age.

When a thrombokinase solution was subjected to one adsorption with barium sulfate, it lost most of its capacity to accelerate the activation of prothrombokinase, and also most of its power to activate prothrombin. Control tests showed that a treated thrombokinase solution did not prevent an untreated

solution from exerting its characteristic effects. Neither were these activities hampered by the addition of a buffer-calcium solution which had been treated with barium sulfate.

DISCUSSION

The blood-clotting process has been partitioned into three primary reactions, carried out in three successive test tubes. The separation, although imperfect, has enabled the development of quantitative techniques for estimating thrombokinase and for following the activation of its precursor. With these new techniques, results have been obtained which may be summarized and interpreted as follows:—

1. Prothrombokinase $\xrightarrow{\text{thrombokinase(?)} + \text{Ca}}$ thrombokinase

2. Prothrombin $\xrightarrow{\text{thrombokinase} + \text{Ca}}$ thrombin

3. Fibrinogen $\xrightarrow{\text{thrombin}}$ fibrin

where all precursors are substances closely associated with the plasma globulins and all three reactions are enzymatic. The position of Ca over the arrows is meant to imply only that ionic calcium conditions the reactions.

The activation of prothrombin by thrombokinase followed the course of a theoretical unimolecular reaction, moreover the concentration of thrombokinase determined the initial rate of prothrombin activation. This behavior suggests that it is an enzyme. Even so, thrombokinase appears to be distinct from the fibrinolytic enzyme (plasmin) (Milstone, 1947).

If thrombokinase is produced by a simple autocatalytic reaction, then it should possess not only the power to activate prothrombin, but also the capacity to accelerate the activation of prothrombokinase. Solutions of thrombokinase actually showed both properties. Moreover, solutions of its precursor showed neither, indicating that both functions were later acquired during incubation with calcium. That both were lost when thrombokinase solutions were heated at 60°C. or subjected to adsorption with barium sulfate, is also in harmony with the view that both functions belong to a single entity. Furthermore, a fair mathematical approximation resulted when the activation of prothrombokinase was treated as a simple autocatalytic reaction, with a minute amount of active form present at the start.

Despite this suggestive evidence, it has not been proven that thrombokinase, *per se*, accelerates the activation of its precursor; and other, more complicated, interpretations are still under consideration. The thrombokinase solutions were crude and may have contained other significant components, in addition to a small amount of thrombin. The evidence presented corroborates previous reports that thrombin does not directly catalyze the activation of prothrombin;

but its possible effect on the activation of prothrombokinase remains undetermined. However, the present diagram may represent the basic coagulation mechanism, on which a variety of accelerating or retarding influences can impinge.

The present use of the term, thrombokinase, is in accord with earlier usage in the following respects: Morawitz (1904) and Mellanby (1909, 1917) associated thrombokinase with protein fractions. Both likened it to enterokinase, and Mellanby considered it an enzyme. Furthermore, they applied the term alike to blood and tissue factors, basing their concept of thrombokinase(s) primarily on function and properties. Rumpf (1913) emphasized that its action was not identical with that of heat-stable lipoid factors. During the present work, experiments not detailed here have suggested that thrombokinase plus calcium activates prothrombin directly, whereas lipoid thromboplastin plus calcium does not. Lipoid thromboplastin seems to hasten coagulation in some other manner.

Prothrombokinase is a logical name for the precursor, and the most widely recognized. Lenggenhager (1936) wanted to replace it with prothrombokinin, because he felt the latter would not carry enzymatic implications to which he objected. Widenbauer (1943) and Reichel (1944) used prothrombokinase, Astrup (1944) prokinase. Laki (1944) and Quick (1947*a*) introduced new terms, but indicated that they were equivalent to the older ones. In addition, factor V (Owren, 1947), prothrombin A (Quick, 1947*b*), labile factor (Quick, 1947*b*), and other designations have recently been applied to what seems to be the same principle.

The problems involved in charting the production of thrombokinase have been attacked with different techniques by Laki (1943) and Owren (1947), both of whom concluded that the autocatalytic phenomenon was concerned with the production of an activator.

The distinguishing features of the present analysis are that the procedure is carried out in three steps that correspond directly to the three theoretical stages, and that thrombokinase and thrombin are separately estimated, each in terms of its defining property. It is believed that this type of analysis permits more secure interpretations and furnishes the groundwork on which more precise methods can be built.

SUMMARY

1. Blood-clotting mechanism has been analyzed by a procedure which devotes a separate experimental step to each of the three primary reactions:

1. Prothrombokinase → thrombokinase
2. Prothrombin → thrombin
3. Fibrinogen → fibrin

2. Activation of prothrombin by thrombokinase followed the course of a unimolecular reaction, and the concentration of thrombokinase determined the initial rate. By this relation thrombokinase was measured, and the activation of its precursor was charted.

3. When the activation of prothrombokinase was plotted against time, the experimental points fell close to the theoretical curve for a simple autocatalytic reaction. Moreover, the process was accelerated by seeding with a small amount of crude thrombokinase. It was concluded that the activation of prothrombokinase involves an autocatalytic or chain reaction.

4. The three-stage procedure made possible the separate estimation of the power to activate prothrombin, on one hand, and the capacity to accelerate the transformation of prothrombokinase on the other. Drastic losses of both activities occurred when crude thrombokinase solutions were heated at 60°C., or adsorbed with barium sulfate.

5. The concentration of calcium was important for the normal progress of prothrombin activation, and also for the transformation of prothrombokinase.

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